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Irradiation of the isolated bovine retinal pigment epithelium with 430 nm light at 20 mW/cm^2 inhibited the unidirectional flux of leucine, glutamate and chloride in the retina to choroid direction; however, this intensity also produced discernible damage to the mitochondria. Reducing the level of radiation did not affect any of the transport systems studied. Ascorbate, morin or vitamin E did not ameliorate the effect of blue light on transport, whereas melatonin did provide protection by forming an effective light filter. Blue light depolarized the transepithelial potential of pigment epithelium, an action spectrum established that a hemoprotein(s) is one mediator of this damage, and potentiated the depolarizing effect. The combination of ethanol and exposure to blue light may constitute a health hazard for humans. Retina-derived factor(s) applied to the apical side of the preparation resulted in a stabilization of the TEP and SCC, followed by a secondary rise in both electrical parameters. These results lead to the hypothesis that the neural retina secretes a factor(s) which is essential for the regulation and maintenance of the RPE under normal physiological conditions and may facilitate repair processes in pathological states.

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FINAL TECHNICAL REPORT FOR AFOSR-87-0189

THE PHOTOTOXICITY OF 'BLUE LIGHT' ON THE FUNCTIONAL PROPERTIES OF THE RETINAL PIGMENT EPITHELIUM

ORIGINAL STATEMENT OF WORK

The phototoxic effect of blue light (435 nm) on isolated pigment epithelium will be investigated. The emphasis will be on functional changes rather than a description of the pathology. The pigment epithelium is analogous to the blood-brain barrier; therefore, the principal functions to be investigated are the integrity of the barrier system and the transport systems known to operate in the pigment epithelium.

Since the damaging effect of blue light on the pigment epithelium is known to be mediated by a photodynamic action, an oxygen-dependent process, the bathing solutions will be aerated with 95% O_2, 5% CO_2. An established ionic solution rather than a media with a serum supplement will be employed to avoid the possible sensitizing effect of molecules in the media/serum.

The tissue will be mounted in a Ussing chamber which permits measurements of the transepithelial potential and short circuit current. From these electrical parameters, the specific resistance can be calculated which is a measure of the "leakiness" of the epithelium. Significant reductions in the specific resistance indicate a failure of the barrier system either by a loosening of the tight junction between epithelial cells or physical damage to the membrane systems. The barrier system will be further analyzed by the employment of radioactive tracers of passive diffusion such as L-glucose. (See Fig. 1)

The unidirectional fluxes in both the retina to choroid (R C) and choroid to retina (C R) direction will be determined for transport systems representative of: 1) facilitated diffusion, 2) Na-dependent active transport systems requiring ATP, and 3) Na-independent concentrating systems which do not require ATP. A minimum of five experiment in each direction (R C and C R) will be performed. The transport of D-glucose will be used as an example of facilitated diffusion and glutamate transport for a Na-dependent active transport system. The L transport system for leucine has been identified in the pigment epithelium and serves as a model for an Na-independent, concentrating transport system with at most a minimal requirement for ATP.

These studies will be first conducted under exposure conditions to blue light which have been established by other investigators to be at or near threshold for pathological changes to occur. It is anticipated that functional changes can be detected before pathological events are observed. If changes in barrier or transport properties are noted at this threshold, the studies will be replicated at a lower intensity of blue light. In any event, an intensity one order of magnitude above the described threshold will also be studied.

When an intensity of blue light has been established to significantly alter any or all of the measures of pigment epithelium function, four agents will be investigated for possible protective or therapeutic effects. This will require pilot studies to ascertain the

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pharmacological dose to be investigated. The agents to be utilized are alpha tocopherol, ascorbic acid, melatonin and quercetin. These will be utilized under two conditions. First, they will be administered only during the exposure period, and then the barrier and transport properties measure. Second, they will be administered immediately after the exposure to blue light to ascertain if any therapeutic action is in evidence.

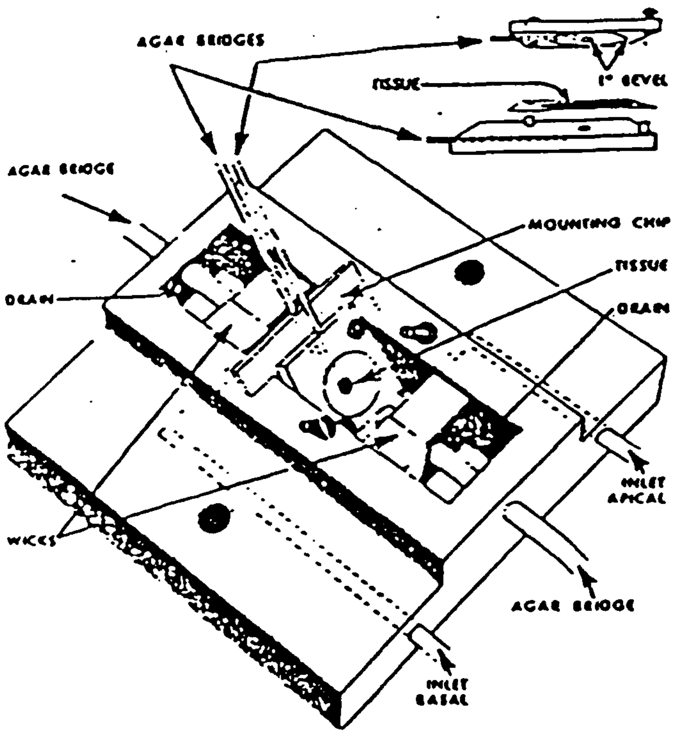

Fig. 1 Schematic view of the Ussing chamber which does not include the thermostatic heating elements.

Summary

It was established that Irradiation of the isolated bovine retinal pigment epithelium with 430 nm light at 20 mW/cm^2 inhibited the unidirectional flux of leucine, glutamate and chloride in the retina to choroid direction; however, this intensity also produced discernible damage to the mitochondria. Reducing the level of radiation of 7 mW/cm^2 did not affect any of the transport systems studied. Administration of Ascorbate, morin or vitamin E did not ameliorate the effect of blue light on the transport, of leucine or glutamate, whereas melatonin did provide protection by mobilizing the melanin granules ~~to form~~ by forming an effective light filter.

It was discovered that Blue light would depolarize the transepithelial potential of the pigment epithelium, ~~The action~~ for an action spectrum established ~~of the depolarizing effect of blue light established~~ that a hemoprotein(s) is one mediator of this damage. ~~of blue light damage to the pigment epithelium.~~ Ethanol (.125%) greatly potentiated the depolarizing effect. of blue light on the transepithelial potential. In addition to mitochondrial damage, it was also noted that blue light irradiation resulted in the appearance of reticulotubular structures in the pigment epithelium. Ethanol (.125%) significantly enhanced the number of tubules when combined with blue light. The combination of ethanol and exposure to blue light may constitute a health hazard for humans.

It was also discovered that retina-derived factor(s) applied to the apical side of the preparation resulted in a stabilization of the TEP and SCC, followed by a secondary rise in both electrical parameters. The late, secondary rise could be eliminated or reduced by the administration of cycloheximide suggesting a dependence upon protein synthesis. The active component(s) is not heat labile and has an apparent molecular weight between 1,000 - 10,000. Electronmicroscopy revealed that after 3 hr of incubation, both control and factor-treated RPE had normal appearing nuclei and mitochondria; however, the factor-treated preparations were significantly thicker (apical to basal membrane) and had more apical processes than the control tissues. These results lead to the hypotheses that the neural retina secretes a factor(s) which is essential for the regulation and maintenance of the RPE under normal physiological conditions and may facilitate repair processes in pathological states.

Report

Our current grant was designed to determine changes in the transport properties of the retinal pigment epithelium (RPE) during exposure to blue light and test specified agents for possible protective effects. Specifica'!y, we established that 20 mw/cm^2 of blue (430 nm) light would inhibit the transport of leucine and glutamate in the apical (retina) to basal (choroid) direction of the RPE. Of the agents tested (ascorbate, melatonin, morin and Vitamin E) only melatonin provided an observable degree of protection in terms of transport. Microscopic studies indicated this probably resulted from the mobilization of melanin granules into the apical process where they functioned as an effective light filter.

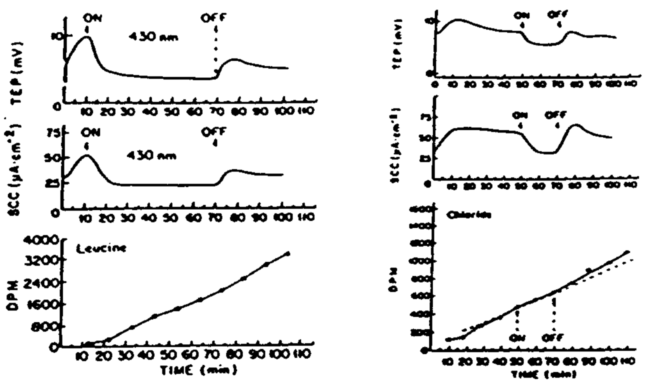

Fig. 1. Blue light irradiation (430 nm) of 20 mW/cm^2 reduces both the TEP and SCC. The slope of the appearance curve is proportional to the unidirectional flux and the dashed line accentuates the change in slope during exposure to blue light. The inhibition of chloride transport in the R-C direction ($P<.01$, $n=6$ each for control and experimental groups) was closely coupled to changes in the electrical parameters. Leucine transport in the R-C direction was reduced by 20 mW/cm^2 of blue light (430 nm) irradiation ($P<.01$, $n=15$ each for control and experimental groups) but occasionally required 10-30 min of exposure for the inhibition to be manifested.

While pursuing the specific aims of the original proposal, a number of significant observations were made which provided direction into investigating the cellular events underlying the damaging effect of blue light on ocular tissue. Because of these developments, the experiments involving the effects of blue lights on glucose transport were not completed. However, we did determine the effect of blue light on chloride transport which was not designated in the original proposal. The unidirectional flux of chloride was

significantly inhibited in the retina to choroid (R-C) direction. As shown in Fig. 1, we found that blue light depolarizes the transepithelial potential (TEP) with an accompanying increase in specific resistance (SR). That is, the short circuit current (SCC), proportional to the TEP, is most significantly reduced. Utilizing the depolarizing effect of blue light, an action spectrum (see Fig. 2) was obtained which corresponds well with the absorption spectrum of cytochrome c oxidase and excludes riboflavin, melanin and retinol as sensitizing agents. By comparing our action spectra with other data, it appears that a different mechanism is involved in the damage produced by UV-A as compared to blue light (see Fig. 3).

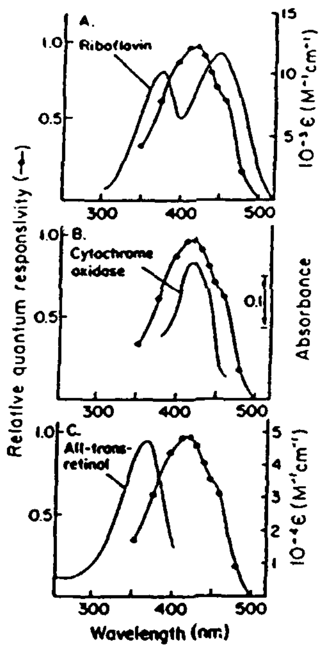

Fig. 2. The action spectra obtained from the depolarization effect of light on the TEP is compared to the absorption spectra of photosensitive molecules present in the RPE. Each point is an average of measurements made on 12 different preparations. The absorption spectrum of riboflavin and retinol do not correspond to our action spectrum whereas the absorption spectrum of cytochrome c oxidase is in close agreement. The absorption spectra were redrawn from the following sources: riboflavin (Otto MK et al, 1981); retinol (Hubbard R, 1956); cytochrome c oxidase (Yoshikawa S & Caughey W, 1982). The cytochrome c oxidase was partially reduced by adding one electron equivalent of NADH/2 heme A.

Blue light is known to damage, either reversibly or irreversibly, cytochrome c oxidase in other cell types as well as beef heart mitochondria (22-24). We verified the inhibition of cellular respiration by blue light in isolated RPE cells by measurement of O_2 utilization. We established through electron microscopy that one consistent sign of structural damage in response to blue light irradiation is to the mitochondria. The damage progresses during the post-irradiation period from the appearance of blisters to a swollen, disorganized state. However, there is more to blue light damage than interference with mitochondrial function. For example, metabolic inhibitors of respiration enhance leucine transport in the R-C direction, whereas blue light has an inhibiting effect. Furthermore, we have recently shown that exposure to 20 mW/cm^2 of blue light for 60 min induces the appearance of tubular structures which appear in the form of bundles. The diameter is somewhat larger than that of microtubules, but may represent the formation of tubuloreticular structures observed in a variety of pathological processes in other cell types (13,21) (see Fig. 4).

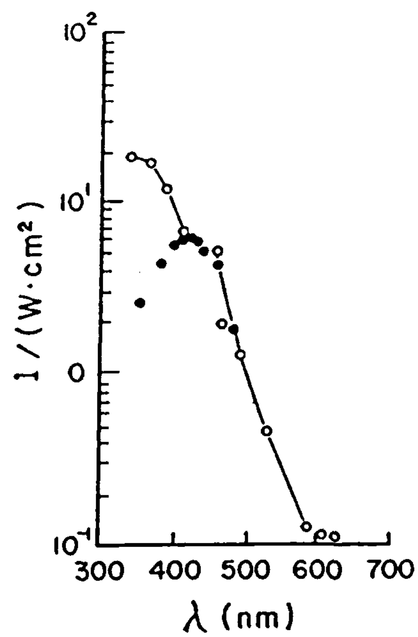

Fig. 3. The short wavelength action spectrum (-o-) for minimal damage to rhesus retina after 100 sec exposure derived by Ham et al (Amer J Ophthal, 1982) is compared to our action spectrum (-••-). The point at 460 nm of Ham's data was used as a reference and the remainder of our action spectrum plotted on a relative basis. There is close agreement between 400-500 nm but a significant departure is noted at the shorter wavelengths.

Fig. 4A. Photomicrograph (16,200 X) of 3-hour control bovine retinal pigment epithelium (RPE). Note numerous mitochondria and abundant smooth endoplasmic reticulum.

Fig. 4B. Photomicrograph (16,200 X) of bovine RPE exposed to 60 min blue light (415 nm), followed by a 30-min recovery period. In addition to mitochondrial damage, there are abundant intracytoplasmic tubules (41.0 $\pm$ 5.6 nm in diameter) throughout the cytoplasm in cross-section and longitudinal section (arrowheads).

We have also demonstrated (see Fig. 5) that in addition to the TEP depolarization by blue light, there is another component which tends to maintain the TEP. Ethanol as low as 0.12% significantly enhances the TEP depolarization by blue light. This appears to be accomplished by the selective reduction of the second component, thus expressing the full magnitude of the blue light-induced TEP depolarization. In order to obtain additional information on this phenomenon, microelectrodes were utilized to determine the electrical responses of the apical and basal membranes to ethanol and blue light. Ethanol (0.5%) alone hyperpolarizes both the apical and basal membrane with a more pronounced effect on the apical membrane potential. Blue light, after the administration of ethanol produces a large depolarization of both the apical and basal membranes again with a greater emphasis on the apical membrane. The basis of the compensatory response is still obscure and would require more extensive study for elucidation. Nevertheless, if these relatively low concentrations of ethanol also potentiate cellular damage by blue light, a possible health hazard is suggested.

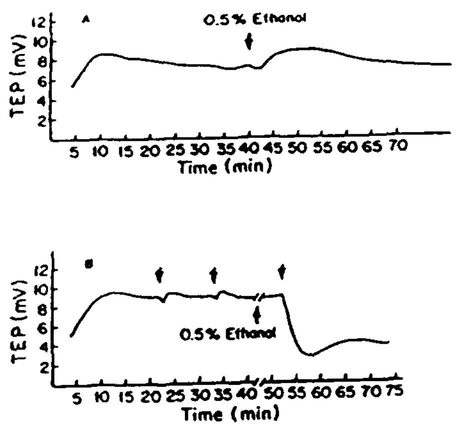

Fig. 5A. Administration of 0.5% ethanol to both RPE surfaces results in a transient reduction in the TEP followed by an increase which requires 10-15 min to attain its maximum value and then returns to about the initial level.

Fig. 5B. Two responses of the TEP to 2 min test flashes of 30 mW/cm^2 blue light (420 nm) are shown. There is an initial depolarization followed by a recovery which occurs during the period of irradiation. Approximately 10 min after the administration of 0.5% ethanol, the test flash produces a pronounced depolarization with only partial recovery.

Factors have been derived from the bovine retina which stimulate growth in a variety of cell types *in vitro* (2,3,7,9,10,14-16). Acidic and basic fibroblast growth factors obtained from retina have been purified and their structures determined (3). In ocular tissues, fibroblast growth factors are mitogenic for capillary and corneal endothelial cells (16.17) as well as lens (15) and pigment epithelial cells (20). Neural retina cells also synthesize mRNA for transforming growth factor-α (TGF-α) and epidermal growth factor (EGF) (11), and the retinal pigment epithelium (RPE) may represent a target organ for these factors (11,17,20). While most studies have emphasized mitogenic activity, it is well known that growth factors can exert a regulatory activity on fully differentiated cells (18). The role of growth factors in inflammation and repair has been actively studied (26). Epidermal growth factor is believed to be a potentially useful agent for the clinical treatment of severe burns, corneal wounds and ulcers (18).

In addition to growth factors, the interphotoreceptor retinol binding protein (IRBP) is also present in the interphotoreceptor matrix (IPM) of bovine retina (4,25) as well as dopamine (12) which acts as a paracrine messenger to the RPE. In view of the abundance of many bioactive substances, we decided to investigate the effects of retina-derived factors (RDF) on the electrical and ultrastructural properties of the bovine RPE. Initially, we employed two techniques for obtaining retina washes containing RDF. The first was taken from a study on retina-derived stimulators of RPE cell proliferation (7) and the second as a procedure to obtain IPM for the isolation of IRBP (4,5,23). Administration of each of these washes equated for total protein resulted in a dramatic stabilization and subsequent rise in the TEP and SCC of the isolated bovine RPE during a 3 hr period of observation. See Figures 6, 7 & 8.

The results show that retina-derived factors obtained by two different procedures applied to the apical side of the RPE will stabilize the TEP and SCC followed by an increase in both electrical parameters. The procedure utilized by others to obtain IPM was just as effective as the method of prolonged soaking of retina in Hank's BSS. Analysis of IPM has shown it to be rich in proteins, containing a limited set of these molecules including the IRBP (1). However, the studies we are aware of did not specifically test for peptides less than 18,000-20,000 molecular weight. In addition to protein, the IPM also contains mucopolysaccharides. Berman (5) has concluded that the matrix components may be derived from many sources and surmised that the glycosaminoglycans (GAG) may be

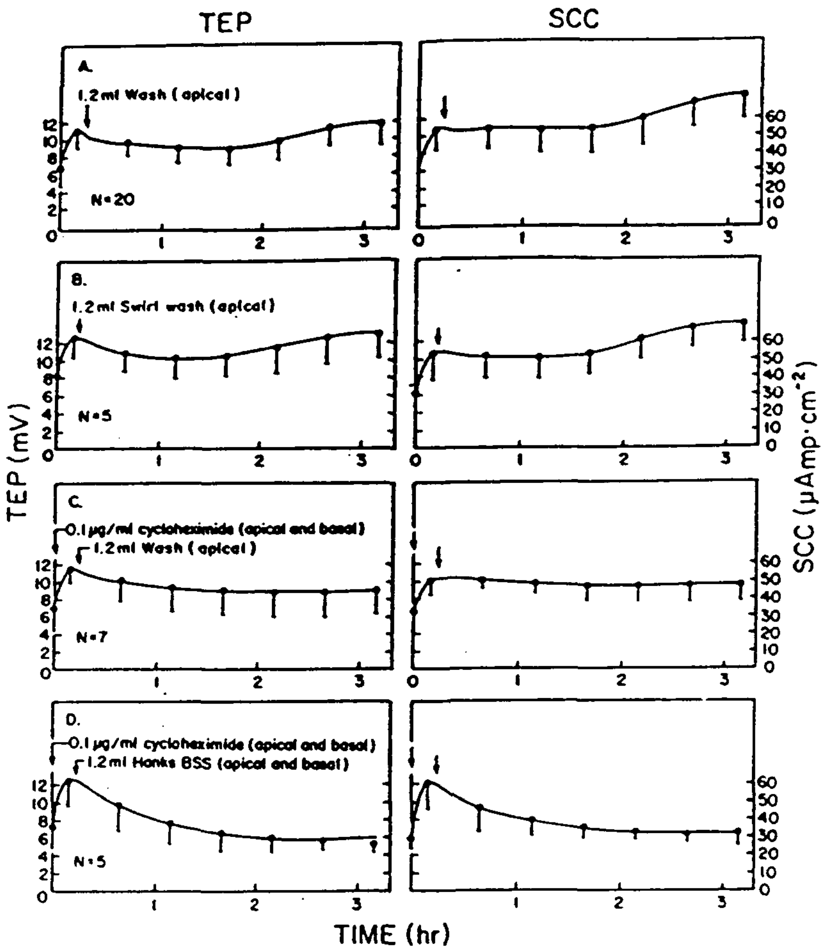

Fig. 6. Data obtained from the two washing procedures are shown in 2A and B. Treatment of tissues with .1 μg/ml cycloheximide eliminates the late secondary rise in TEP and SCC observed in preparations receiving the retina wash alone. The differences in TEP and SCC between the wash-treated versus wash and cycloheximide are significant ($P<.01$) according to Student's t test when analyzed at 190 min. There is no significant difference prior to the 2 hr incubation time. The TEP and SCC of the preparations receiving cycloheximide and retina wash (2C) were also significantly ($P<.01$) greater than the tissues receiving cycloheximide alone when analyzed at 2 and 3 hr of incubation. Cycloheximide did not significantly alter the TEP and SCC of the preparation (2D) not receiving the wash.

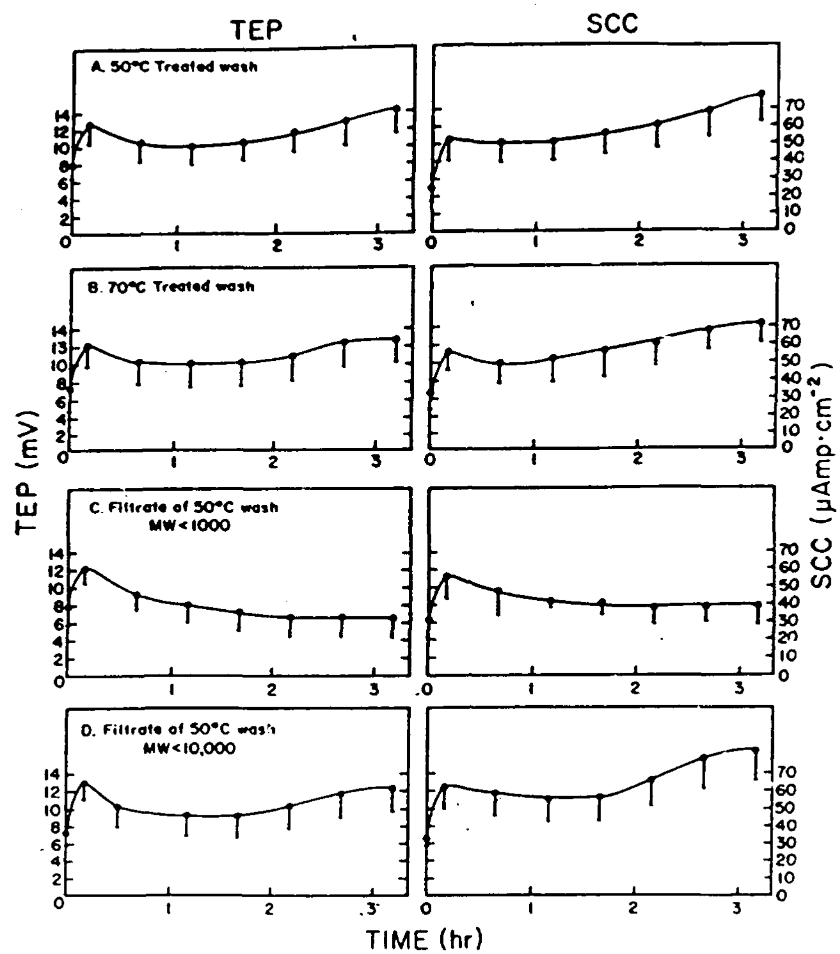

Fig. 7. Heating the wash to 50°C or 70°C for 30 min did not alter its bioactivity as shown in 5A and B. Filtrate in which molecules greater than 1000 molecular weight were excluded did not reveal any bioactivity in terms of affecting the TEP and SCC (5C). When molecules with molecular weight greater than 10,000 were excluded, the TEP and SCC did not differ from treatment with unfiltered wash (5D).

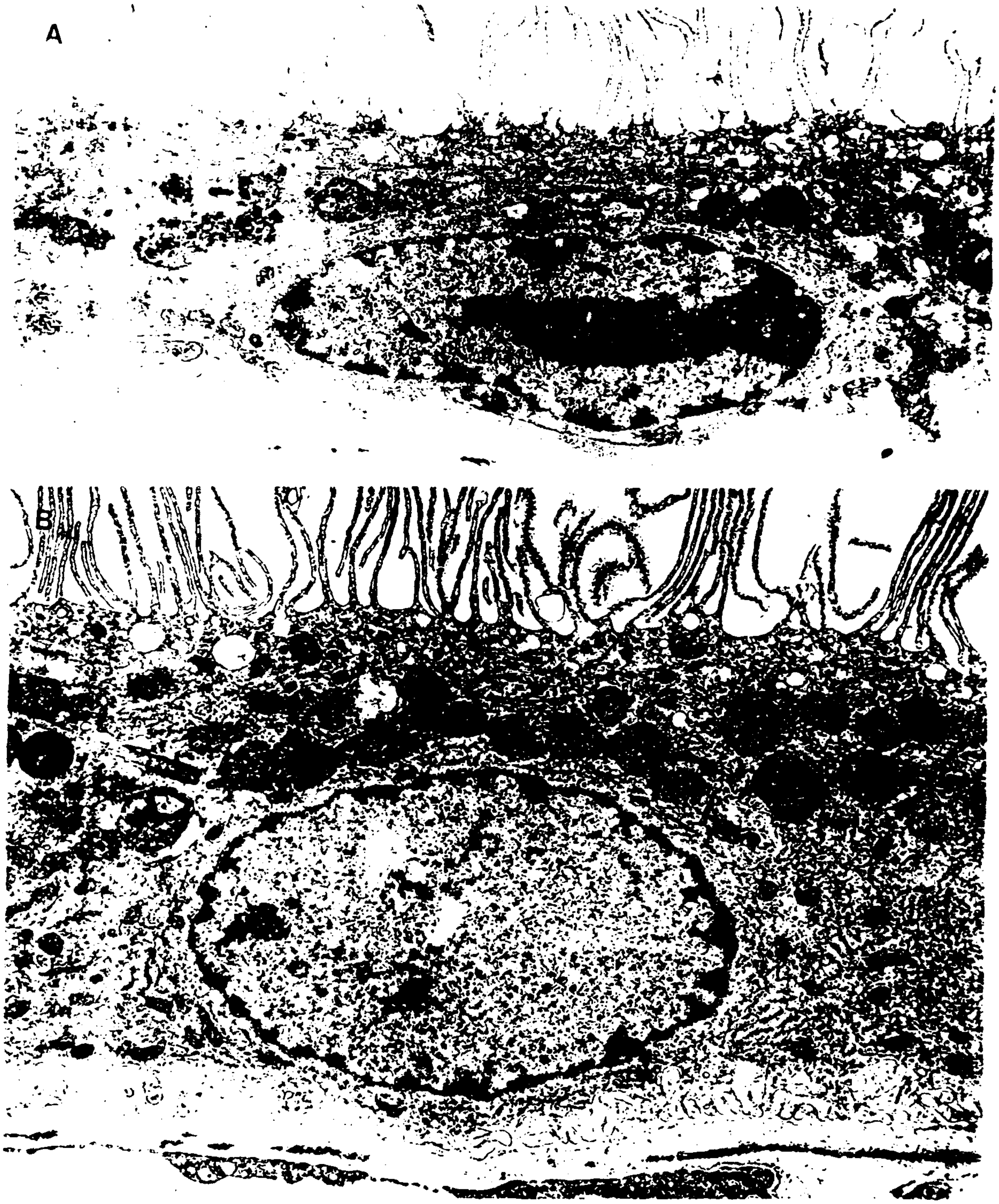

Fig. 8. Representative micrographs of control and wash-treated RPE are shown in 8A,B, respectively. Analyzing 5 sections from each of three RPE tissues (N=15) for both control and wash-treated preparations revealed average thicknesses of 5.0 $\pm$ .5 and 8.1 $\pm$ .9μ, respectively (P<.01). The number of apical processes per 10 μ of apical surface was greater in the wash-treated RPE (37.9 $\pm$ 7.8) than the control (20.4 $\pm$ 4.4) tissues. Similarly, significantly (P<.01) more vacuoles in a 10 x 1 μ area adjacent to the apical membrane were noted in the control (3.7 $\pm$ 1.6) than in the wash-treated preparations (1.5 $\pm$ .9).

synthesized by both photoreceptors and RPE but that IRBP originate in the photoreceptors. The low molecular proteins and glycoproteins may be provided by the pigment epithelium. Since there is some uncertainty as to source of components in IPM, we cannot be sure that the bioactive factors in our washes are of neural retina origin. However, the selectivity of the apical membrane does suggest that specific receptors or sites of action reside in this membrane. The failure of the basal membrane to respond to the administration of retina wash does not appear to result from the physical barrier provided by the attached tapetum and choroidal remnants. For example, we have observed effects of micromolar quantities of succinate on leucine transport when applied to the basal side of the RPE preparation which were equal or even superior to application to the apical surface. It is possible that the choroidal remnants may represent an enzymatic barrier in which a fraction of materials are degraded as they pass through the choroidal tissue. However, doubling the quantity of wash on the basal side (not shown) did not elicit any enhancement of the TEP and SCC so it is likely that a difference in sensitivity to the retina wash exists between the apical and basal membranes.

The elimination or reduction of the late secondary rise in the TEP and SCC by the administration of cycloheximide suggests this process is dependent upon protein synthesis. Control studies indicated that the level of cycloheximide employed was not detrimental to the TEP and SCC and the effect on the secondary rise in TEP and SCC does not represent a general toxicity. At this stage of investigation, we cannot be sure that the presumed protein synthesis was initiated by specific signals or that it represents a permissive effect resulting from stabilization of the membrane systems. The enhancement of the TEP and SCC by delayed (2-3 hr) administration of the retina wash indicates a rather direct or immediate effect on the electrical parameters of the RPE (see Fig. 9). The electronmicrographs clearly show the beneficial effects of the wash. The apical processes are more dense with fewer underlying vacuoles, and the thickness of the RPE is greater in the wash-treated tissue as compared to controls. In general, the ultrastructure of the wash-treated RPE is remarkably similar to that of freshly prepared cow eyes (6).

The active RDF(s) in the retina wash are not heat labile, at least with treatment up to 30 min at 90C. Exclusion filtration indicates a low molecular weight somewhere in the range of 1,000-10,000. These properties exclude the fibroblast growth factors and IRBP as the active agents but are consistent with TGF-α and EGF in terms of molecular weight and

stability. However, administration of these factors did not elicit the stabilization and secondary rise of the TEP in the bovine RPE. Regardless of the identity of the bioactive component(s), our studies demonstrate the significant effects of RDF on the electrical and ultrastructural properties of the bovine RPE and provide direction for biochemical and electrophysiological experiments. These results also lead to the hypothesis that the neural retina may secrete a substance which is essential for the regulation and/or maintenance of a normally functioning RPE. This functional relationship has significant implications for the pathophysiology of the retina and suggests the possibility of enhancing repair processes in the RPE as a result of photodamage.

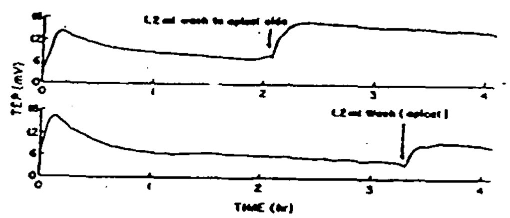

Fig. 9. Sample recordings of the TEP which depict the effects of delayed administration of retina wash to the apical side. The responsiveness of the TEP to wash treatment declines as the delay period is prolonged. A total of five preparations were studied in which the wash was delayed for 2 hr in three tissues and delayed for 3 hr in the other two.

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Pautler, E.L. and Beezley, D. The effects of ethanol and "blue light" on the retinal pigment epithelium.

Pautler, E.L. and Beezley, D. α-1 adrenergic effects on the bovine retinal pigment epithelium.

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